Structure
Ways & Means

Reverse Micelle Encapsulation of Membrane-Anchored Proteins for Solution NMR Studies

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SUMMARY

Perhaps 5%–10% of proteins bind to membranes via a covalently attached lipid. Posttranslational attachment of fatty acids such as myristate occurs on a variety of viral and cellular proteins. High-resolution information about the nature of lipidated proteins is remarkably sparse, often because of solubility problems caused by the exposed fatty acids. Reverse micelle encapsulation is used here to study two myristoylated proteins in their lipid-extruded states: myristoylated recoverin, which is a switch in the Ca^{2+} signaling pathway in vision, and the myristoylated HIV-1 matrix protein, which is postulated to be targeted to the plasma membrane through its binding to phosphatidylinositol-4,5-bisphosphate. Both proteins have been successfully encapsulated in the lipid-extruded state and high-resolution NMR spectra obtained. Both proteins bind their activating ligands in the reverse micelle. This approach seems broadly applicable to membrane proteins with exposed fatty acid chains that have eluded structural characterization by conventional approaches.

INTRODUCTION

Covalent attachment of fatty acids such as myristate and palmitate occurs on a wide variety of viral and cellular proteins (Resh, 1999). Myristate, a 14 carbon saturated fatty acid, and palmitate, a 16 carbon saturated fatty acid, commonly serve as key elements of membrane targeting and anchoring of proteins. Reversible membrane binding, controlled through triggered exposure of lipid anchors, is central to protein-protein interactions mediating signal transduction at the membrane (Casey, 1995). Lipid anchors are also now thought to be critical to the exit of viruses from the eukaryotic cell (Maurer-Stroh and Eisenhaber, 2004). For example, the coupled interaction of fatty acids covalently attached to the HIV matrix protein with the membrane and phosphoinositides embedded in target membranes is thought to be central to its localization to the plasma membrane, which in turn begins the assembly of the immature virus (Ono et al., 2004; Saad et al., 2006). Accordingly, this initiation of virion assembly is argued to be a prime candidate for pharmaceutical intervention (Lindwasser and Resh, 2002). Structural knowledge of this process would be of obvious utility. However, detailed characterization of the structural features of the HIV-1 myristoylated matrix protein (MA[+myr]), both by itself and in complex with phosphatidylinositol-4,5-bisphosphate (PI[4,5]P_2), has been frustrated by the poor solution properties of both the MA(+myr) protein and PI(4,5)P_2 (Saad et al., 2006). Often what is done is to simply remove the portion of the protein that carries the lipid anchor, leaving a highly soluble and well-behaved protein. This, however, obviously precludes examination of the role of the anchor itself and the interaction of the anchored protein with molecules presented by the membrane. A review of the Protein Data Bank (PDB) reveals that high-resolution information about the nature of lipidated proteins is remarkably sparse, perhaps because of this inherent difficulty. The classic and, one might argue, only structurally well-defined lipid anchor switch is that of recoverin, which shows a calcium-dependent exposure of a modified myristoyl group (Ames et al., 1997; Tanaka et al., 1995). Recoverin(+myr) is a membrane-associated protein that reacts to the influx of Ca^{2+} to extrude the hydrophobic myristoyl chain into the rod outer segment membranes. The structural characterization of myristoylated recoverin (recoverin[+myr]) in the Ca^{2+}-saturated state has also proved elusive due to the limitations imposed by aggregation.

In an effort to overcome many of the difficulties presented by the poor solution behavior of lipidated proteins in the membrane-anchored state, we have adapted the reverse micelle as a surrogate for the membrane bilayer in order to enable high-resolution structural studies by solution NMR methods. Over the past decade there has been significant progress in the preparation of soluble protein molecules encapsulated within the protective water core of a reverse micelle dissolved in a low-viscosity solvent such as the liquid alkanes (Peterson et al., 2005a, 2005b; Wand et al., 1998). The low-viscosity solvent allows for sufficiently fast tumbling of the reverse micelle particle to significantly improve the NMR relaxation properties of the protein (Peterson et al., 2005a; Wand et al., 1998). This new approach is illustrated with the two myristoylated proteins discussed

above: recoverin(+myr) and the HIV-1 MA(+myr). Both proteins have been successfully encapsulated in the lipid-extruded state and high-resolution NMR spectra have been obtained. Both proteins can be directly titrated with their natural activating ligands in the reverse micelle and their interactions followed by high-resolution NMR. In the case of the HIV-1 MA(+myr), chemical shift mapping identifies the location of the $PI(4,5)P_2$ binding site.

RESULTS

Encapsulation of myristoylated proteins within a surfactant system solvated in an alkane solvent proceeded as suggested by encapsulation protocols for water-soluble proteins (Babu et al., 2003; Lefebvre et al., 2005; Peterson et al., 2005b; Shi et al., 2005; Wand et al., 1998). The caveat for recoverin(+myr) and the HIV-1 MA(+myr) protein is the behavior of the hydrophobic myristoylate anchor. In free aqueous solution, both proteins show ligand-dependent exposure of the covalently attached myristoyl group, which generally complicates the solution behavior of both proteins in the putative membrane-anchored state where the myristoyl group is extruded. The binding of calcium to recoverin(+myr) results in exposure of the otherwise buried myristoyl group (Ames et al., 1995, 1997; Tanaka et al., 1995). Similarly, the specific binding of $PI(4,5)P_2$ to the HIV-1 MA(+myr) results in extrusion of the largely sequestered myristoyl group (Saad et al., 2006). It is predicted that the reverse micelle surfactant shell will provide a solubilizing environment for the myristoyl group and will therefore compete with the hydrophobic interior of the soluble protein for the lipid group, even in the absence of the ligand (Figure 1). This is indeed the case for both proteins.

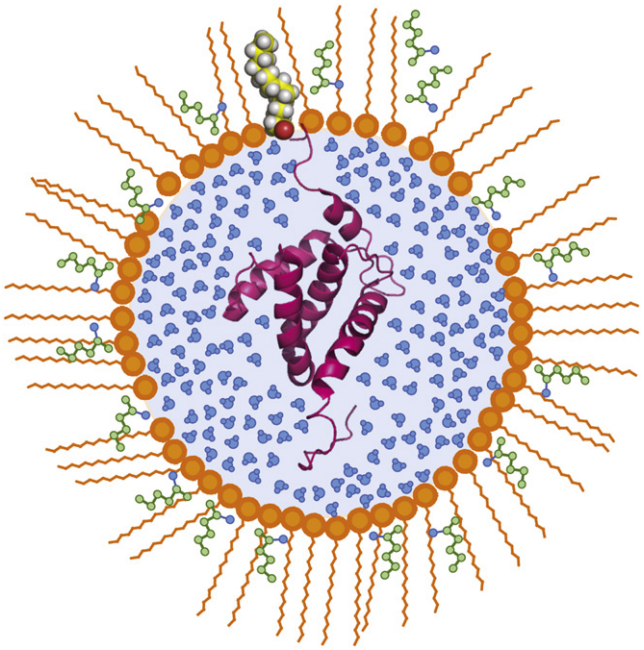

Figure 1. Reverse Micelle Encapsulation of Lipidated Proteins for Solution NMR

Schematic illustration of the structure of a reverse micelle surfactant assembly hosting a lipidated protein. A ribbon representation of the NMR structure of HIV-1 MA(−myr) (Doyle et al., 1998) is shown. The surfactant CTAB (orange) cosolubilizes with hexanol (green) to form the reverse micelle shell. The myristoylated protein HIV-1 MA(+myr) inserts into the aqueous interior with the acyl chain extruded into the hydrophobic surfactants. The reverse micelle particle is solvated in a compatible low-viscosity solvent such as the short-chain alkanes (Wand et al., 1998). This sample is designed to avoid the poor solution behavior of lipidated proteins that often defeats detailed solution NMR studies.

Encapsulation of Recoverin(+Myr)

We were unable to find conditions for direct encapsulation of calcium-saturated recoverin(+myr), perhaps because Ca^{2+}-bound recoverin in solution tends to preaggregate, which might prevent subsequent encapsulation. In the absence of calcium in free solution, the myristoyl tail is sequestered and the protein is well behaved. The maximum concentration tolerated was ~2 mM before aggregation of the myristoylated protein was evident. The identification of optimal encapsulation conditions for recoverin(+myr) began with the selection of the cetyltrimethylammonium bromide (CTAB) and hexanol surfactant system (Lefebvre et al., 2005) as the most probable success for a myristoylated protein, with an acidic pI of 5.3. The preliminary results indicated mixed states of the encapsulated recoverin(+myr), likely resulting from myristoyl-extruded and myristoyl-sequestered forms. The aqueous protein buffer conditions were modified to include 1 mM ethylene glycol tetraacetate (EGTA) to ensure removal of any free Ca^{2+} ions from the solution, thereby selecting the myristoyl-sequestered state and limiting any protein aggregation. The concentration of NaCl was increased to 100 mM to further reduce the propensity to aggregate at the high protein concentration used. Encapsulation of the protein in this buffer system into CTAB/hexanol reverse micelles resulted in a largely disordered state (Figure 2B). The spectrum is characterized by broad crosspeaks of extremely low intensity, indicative of a dynamic equilibrium of a mixture of states that undergo exchange on the chemical shift timescale. Addition of Ca^{2+} to the encapsulated recoverin by direct injection of a concentrated solution of calcium chloride results in a more homogeneous protein state, apparently due to stabilization by the binding of Ca^{2+} to the protein (Figure 2). There is no evidence of aggregation in the reverse micelle encapsulated state of the Ca^{2+}-bound recoverin(+myr). Final optimizations were directed at maximizing protein concentration in the reverse micelle. Surfactant concentration, aqueous solution protein concentration, and water loading are the adjustable parameters. The total reverse micelle assembly size (correlation time) is also a consideration for spectroscopic success. The best signal-to-noise spectra were obtained with 200 mM CTAB, a starting protein concentration of 2.2 mM, and a water loading, W_o, of 25. The resulting protein concentration in the reverse micelle solution was 180 μM recoverin(+myr). The pentane solution of encapsulated calcium-saturated recoverin is stable for months at room temperature. Other surfactant systems examined included the triple-surfactant system lauryldimethylamine oxide (LDAO), dodecyltetraethylene glycol ether ($C_{12}E_4$), and sodium bis(2-ethylsulfosuccinate) (AOT) (Peterson et al., 2005b). This surfactant system was successfully applied to the encapsulation of flavodoxin (Lefebvre et al., 2005), which also has a low pI, but failed to successfully encapsulate recoverin(+myr) in a folded state.

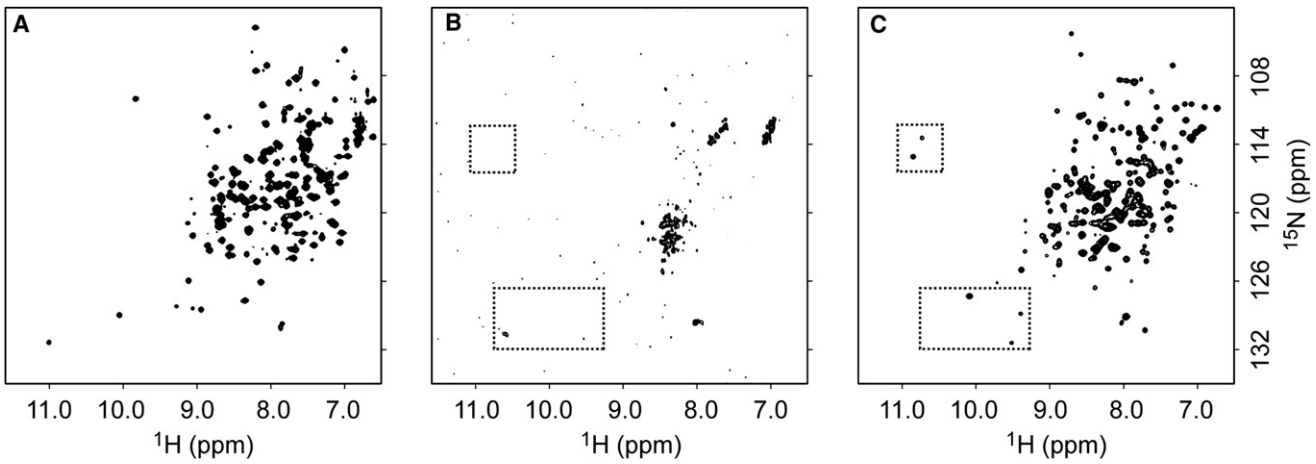

Figure 2. Encapsulation of Myristoylated Recoverin

^{15}N HSQC spectra of (A) recoverin(+myr) in aqueous buffer in the Ca^{2+}-free apo state; (B) Ca^{2+}-free recoverin in CTAB/hexanol reverse micelles; and (C) Ca^{2+}-bound state of recoverin in CTAB/hexanol reverse micelles. The spectra in (B) and (C) are the identical sample without and with Ca^{2+} collected with the same number of transients; the contour threshold is five times lower in (B). The increased sensitivity and spectral dispersion of the Ca^{2+}-bound state in reverse micelles is a clear demonstration of the folding of encapsulated recoverin(+myr) with the addition of Ca^{2+}. The boxes include crosspeaks that are followed during the titration of Ca^{2+} into reverse micelles shown in Figure 3.

The encapsulated apo state of the protein can be quantitatively titrated with calcium and recovers an ^{15}N HSQC spectrum consistent with the native structure in free solution. Notably, the very low intensities of crosspeaks in the spectrum of the apo state are converted to a high-intensity spectrum having the anticipated signal-to-noise ratio (Figure 2). This unusual result is most consistent with the apo state being composed of a dynamic mixture of structures (including lipid-sequestered and -extruded forms) that interconvert on the chemical shift difference timescale. This effect is nicely illustrated by two glycine residues, G79 and G115, that participate in the turn region of the calcium-loaded EF hands and have two characteristic upfield shifted amide N-H correlations in the calcium-saturated state. These crosspeaks can be used to follow the binding of calcium to the protein (Figures 3A–3E). Binding of calcium stabilizes the form of the protein with an extruded lipid group (Ames et al., 1997). At a Ca^{2+}:recoverin(+myr) stoichiometry of 2:1, weak signals begin to appear for these two glycine residues and increase upon subsequent addition of calcium while their chemical shifts remain constant. This demonstrates that the Ca^{2+}-binding exchange rate is much slower than the chemical shift difference timescale and is therefore consistent with high-affinity and functional Ca^{2+} binding under these conditions (see also Figure S1 available online).

The behavior of the tryptophan indole N-H chemical shift correlations also inform on the extensive dynamic averaging in the encapsulated apo state of the protein. In the absence of calcium, there is a single weak signal due to one or more indole N-H correlations of W31, W156, and W104. We also ascribe this residual, broad signal to the heterogeneous mixture of structures of the encapsulated apo state of recoverin(+myr) that interconvert on the chemical shift difference timescale (Figures 3F–3J). As calcium is added, this broad crosspeak disappears and three sharp indole N-H crosspeaks arise. The indole N-H crosspeak of W31 is the first to be sufficiently populated to appear above the noise floor, at a Ca^{2+}:recoverin(+myr) stoichiometry of 1.5:1.0, whereas the indole N-H correlations of W156 and W104 appear at slightly higher calcium concentrations. The intensity initially appears slowly, as the protein competes with the residual free EGTA for the added calcium, then builds up in a linear stoichiometric fashion with added Ca^{2+}. This accounts for the greater than stoichiometric 2:1 Ca^{2+}:recoverin(+myr) plateau of the titration curve at 7.5:1 Ca^{2+}:recoverin(+myr) (Figure 3). An endpoint of the titration is reached where further addition of calcium does not result in additional signal intensity. At very high calcium concentrations, corresponding to a greater than 10:1 Ca^{2+}:recoverin(+myr) molar ratio, the excess calcium apparently disrupts the stability of the reverse micelle assembly and a catastrophic loss of sample integrity ensues.

Encapsulation of the HIV-1 MA(+Myr)

Encapsulation conditions have been developed for the N-terminal myristoylated matrix protein which mediates membrane binding by an allosteric $PI(4,5)P_2$-dependent myristoyl switch mechanism (Saad et al., 2006). Direct determination of the structure of the native $PI(4,5)P_2$-bound state of the MA(+myr) domain has been precluded by insolubility problems and by the tendency of native $PI(4,5)P_2$ to form high molecular weight aggregates in solution. Recombinant MA proteins that contain (MA[+myr]) or lack (MA[−myr]) the N-terminal myristoyl group were encapsulated into reverse micelles using the CTAB/hexanol surfactant system in pentane. The HIV-1 MA(+myr) protein has a basic pI of 9.1. Attempts to encapsulate the HIV-1 MA(+myr) with either the acidic surfactant AOT or a triple-surfactant mixture containing AOT:CTAB:LDAO proved fruitless. Although the MA protein encapsulated, it was unfolded in both surfactant systems. In contrast, the CTAB/hexanol surfactant system produced an encapsulated folded myristoylated protein giving an ^{15}N HSQC spectrum indicative of a folded protein. Encapsulation conditions were then optimized for maximum

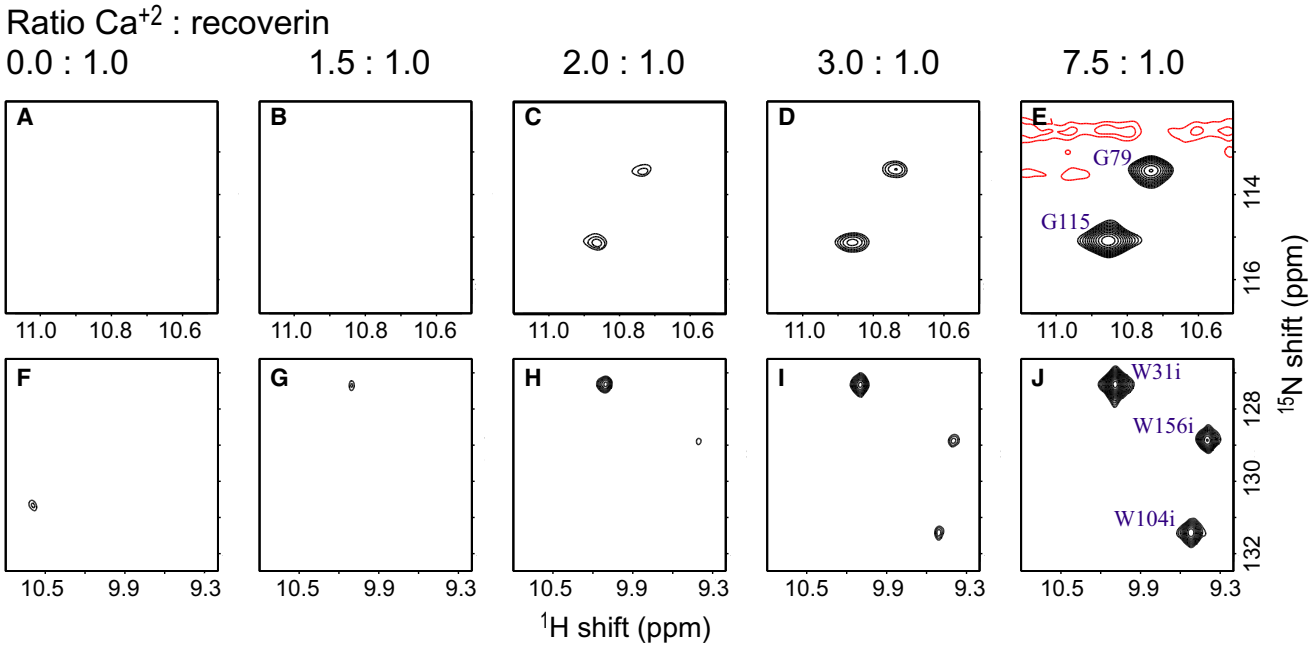

Figure 3. Titration of Recoverin with Ca^{2+} in Reverse Micelles

The ^{15}N HSQC spectra of recoverin(+myr) in CTAB/hexanol reverse micelles in (A)–(E) are titration points following the amide N-H correlations of residues G79 and G115 in the native state with the addition of Ca^{2+}. The molar ratios of Ca^{2+}:recoverin(+myr) are indicated above the panels. (F)–(J) The disappearance of the indole N-H correlations of the tryptophan side chain in the disordered apo state and the appearance of their counterparts in the calcium-saturated state are shown. The molar ratios are the same as in the top panels. The full spectra are shown in Figure S3.

protein concentration. A maximum protein concentration in aqueous buffer of 4 mM could be maintained for a time sufficient for injection. Using a surfactant concentration of 200 mM CTAB and 8.5% (v/v) hexanol and a W_o of 25 gave a final protein concentration of 180 μM. The ^{15}N HSQC spectrum of encapsulated MA(+myr) is shown in Figure 4 and is compared to the aqueous spectrum of MA(+myr) and MA(−myr) in Figure S2. Except for a few resonances associated with surface residues that may have chemical shift perturbations due to electrostatic interactions with the surfactant shell, the backbone amide N-H chemical shifts of the encapsulated MA(+myr) protein are remarkably similar to those of the free-solution MA(−myr) protein (Figure 4), which clearly indicates that the myristoyl group of the former leaves its hydrophobic pocket on the protein when placed into the reverse micelle assembly. The amide N-H chemical shift correlations were therefore initially assigned by reference to the $^{15}N/^{1}H$ chemical shifts reported for the HIV-1 MA(−myr) in free aqueous solution (Massiah et al., 1994) and then corroborated using an ^{15}N NOESY HSQC spectrum of the encapsulated protein and the main-chain directed resonance assignment approach (Wand and Nelson, 1991).

Titration of the MA(+myr) protein with its membrane-targeting ligand 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphoinositol-4,5-bisphosphate in free solution results in degradation of the NMR performance of the protein due to severe line broadening (Saad et al., 2006). As mentioned above, this is due, in part, to the tendency of native $PI(4,5)P_2$ to form micelles in water. $PI(4,5)P_2$ is insoluble in alkanes but is solubilized by CTAB/hexanol reverse micelles. This allows the MA(+myr) to be probed for the binding of the $PI(4,5)P_2$ ligand. The binding of $PI(4,5)P_2$ into the MA(+myr) was followed by ^{15}N HSQC spectra (Figure 5A). Several MA(+myr) amide N-H crosspeaks can be seen to shift upon binding of $PI(4,5)P_2$, but most remain relatively unperturbed (see Figure S3). The continuous shifting of selected resonances indicates specific binding that is in fast exchange on the NMR chemical shift timescale (Figure 5). The titration does not plateau (saturate) at the highest molar ratio of $PI(4,5)P_2$:MA(+myr) of 5.5:1 that could be reached. Unfortunately, the reverse micelle assembly becomes unstable at higher concentrations of $PI(4,5)P_2$. The amide sites that show significant shifts are indicated in red in the backbone ribbon diagram of the HIV-1 MA(−myr) NMR structure of Summers and coworkers (Massiah et al., 1994) (Figure 5B). The binding is clearly localized at the basic surface patch containing the turn connecting helices III and IV, consistent with solution-state studies using soluble $PI(4,5)P_2$ analogs that contain truncated acyl chains.

DISCUSSION

Reverse micelle encapsulation appears to provide a simple and direct route to the detailed characterization of myristoylated proteins in their lipid-extruded membrane-anchored state. Under the surfactant and water compositions used here, the reverse micelles contain on average several thousand water molecules. The quality of the spectra that are obtained for encapsulated calcium-saturated recoverin(+myr) and the HIV-1 MA(+myr) are consistent with only one protein molecule in a reverse micelle. Higher occupancy would lead to severely broadened resonance lines (Wand et al., 1998).

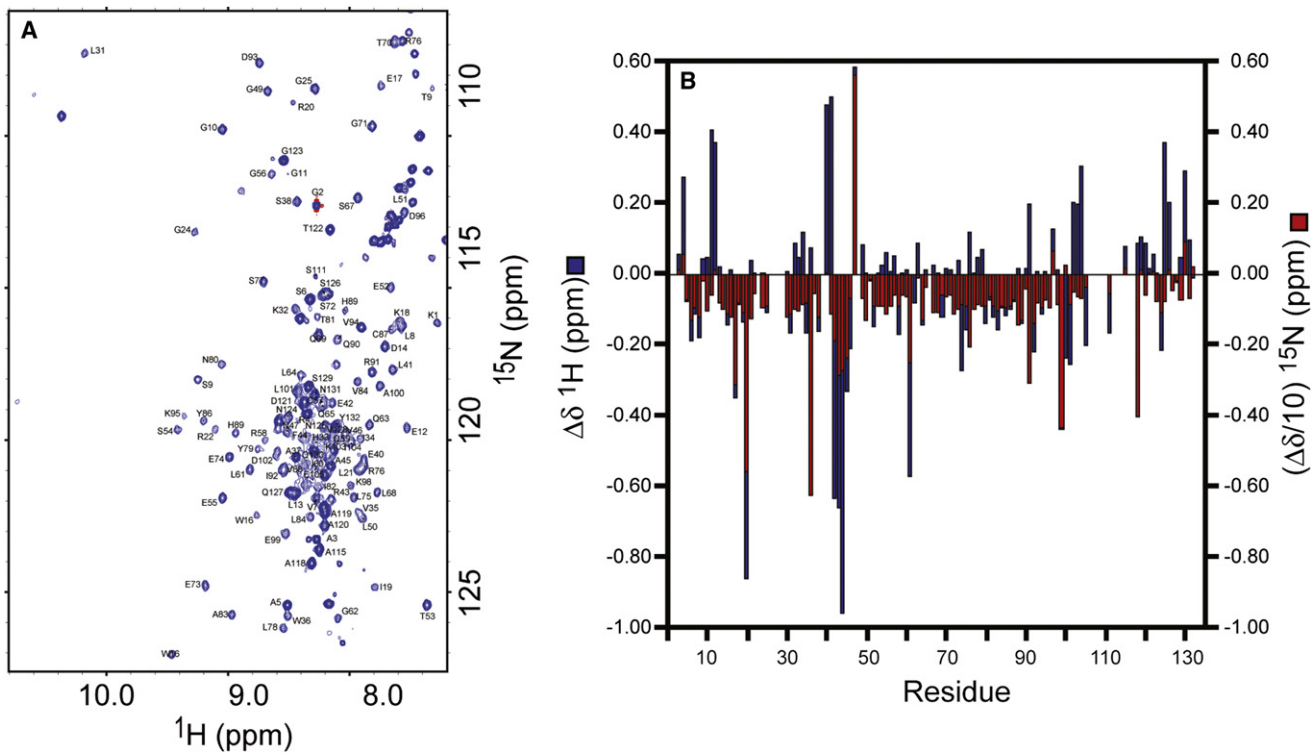

Figure 4. Encapsulation of the Myristoylated HIV-1 Matrix Protein

(A) ^{15}N HSQC spectrum of HIV-1 MA(+myr) in CTAB/hexanol reverse micelles. Assigned amide correlations are labeled.

(B) The chemical shift differences between amide N-H correlations in the aqueous HIV-1 MA(−myr) spectrum and the corresponding resonances in the HIV-1 MA(+myr) protein encapsulated in CTAB/hexanol reverse micelles are plotted. The ^{15}N chemical shift differences have been scaled by a factor of 10 to reflect the difference in gyromagnetic ratios of ^{1}H and ^{15}N. See Figure S4 for overlays of the full spectra.

The use of reverse micelle encapsulation to study lipidated proteins should prove highly complementary to those involving use of aqueous detergent micelles or isotropic bicelles. A particular advantage of the reverse micelle approach is the ability to employ low-viscosity fluids such as liquid ethane that preserve favorable relaxation properties and thereby avoid the necessity for extensive deuteration (Peterson et al., 2005a). On the other hand, the continuing development of the more slowly tumbling isotropic bicelle as a vehicle for studying membrane proteins in a true bilayer lipid environment will provide support for studies undertaken in the reverse micelle single-layer shell (Kim et al., 2009).

The two example proteins studied here have quite distinct biological roles and contrasting ligand triggers for membrane anchoring. Recoverin employs the water-soluble calcium cation to initiate membrane anchoring that subsequently results in activation of the G-protein-mediated signaling cascade. The calcium-activated state of the protein is fully folded while anchored to the reverse micelle surfactant shell. Interestingly, the presumably functionally inappropriate lipid-extruded apo state of the protein is promoted by encapsulation but results in an ensemble of states. The encapsulation of the protein apparently sufficiently modifies the equilibrium between the structures of recoverin(+myr) that sequester or extrude the myristoyl group such that the latter is favored. This generates a dynamic mixture of sequestered and extruded forms in the apo state and suggests that this mixture is dynamically exchanging in the absence of calcium, which would be consistent with the calcium-mediated signaling switch of the protein. It is interesting to note that the encapsulated Ca^{2+}-free recoverin(+myr) exhibits very broad NMR resonances because the dynamic exchange of sequestered and extruded forms of the apo protein causes structural changes that result in chemical shift differences that are not averaged, thereby causing exchange broadening. The confined space of the reverse micelle apparently slows the dynamical interconversion of the ensemble of states such that no single (or average) spectral signature dominates. A similar effect is seen during the process of protein cold denaturation in reverse micelles (Babu et al., 2004; Pometun et al., 2006; Whitten et al., 2006).

The equilibrium between the sequestered and solvent-exposed states of the myristoylated HIV-1 matrix protein is also significantly altered by encapsulation and is pushed toward what is putatively the membrane-anchored structure of the protein. In contrast to recoverin(+myr), however, this state of the myristoylated MA protein is highly structured and is, by chemical shift analysis, closely similar to the unmyristoylated form of the protein, that is, the myristoyl group is fully extruded from the protein. This behavior is consistent with the idea that the MA protein needs to probe the identity of a given membrane by repetitive insertions of the anchor *followed by* binding of the plasma membrane-specific $PI(4,5)P_2$ recognition element. In

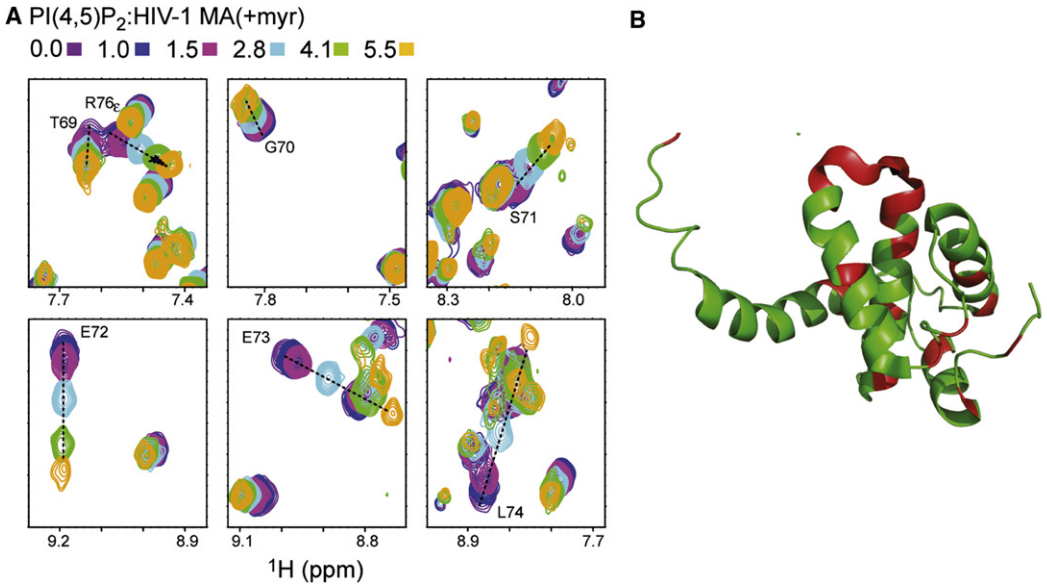

Figure 5. Titration of the Encapsulated HIV-1 MA(+Myr) with PI(4,5)P_2

(A) Expansions of the ^{15}N HSQC spectra of the HIV-1 MA(+myr) in CTAB/hexanol reverse micelles illustrating amide N-H correlations that shift upon titration with PI(4,5)P_2. The molar ratios of PI(4,5)P_2:MA(+myr) are color coded as indicated.

(B) A ribbon representation of the solution NMR structure of the HIV-1 MA(−myr) (Massiah et al., 1994) (PDB ID code 2HMX) is shown with the backbone colored in red at sites that demonstrate significant shifts in the HSQC spectra on titration with PI(4,5)P_2. The figure was drawn with PyMOL (DeLano, 2002). The full spectra are shown in Figure S5.

this model, the protein would naturally be stable and structured in both the soluble sequestered and membrane-anchored extruded states. Whether this distinction persists across the many different lipidated proteins remains an interesting and exciting question.

Analysis of the chemical shift perturbations brought about by the binding of PI(4,5)P_2 to MA(+myr) indicates a similar but not identical interaction surface to that highlighted by the binding of a more water-soluble PI(4,5)P_2 analog (Saad et al., 2006). Detailed structural studies of the encapsulated MA(+myr):PI(4,5)P_2 complex described here are ongoing and will be reported elsewhere.

In conclusion, the results presented here suggest that the reverse micelle particle has been successfully adapted to the study of myristoylated proteins in the lipid-extruded state using high-resolution solution NMR methods. The generality of the encapsulation strategy for studying lipidated proteins remains to be ascertained, especially with respect to the type of membrane anchor employed. Although some limitations are apparent, quite detailed structural investigations are clearly made feasible by this approach and include characterization of the structural basis for the interaction of ligands with proteins in the membrane-anchored state. Both water-soluble and -insoluble ligands can be employed. Although the confined space effects of the reverse micelle complicate quantitative interpretation of observed binding phenomena, the ability to investigate the interactions of lipid-anchored proteins with small ligands in structural detail is a significant and very useful capability. Furthermore, the performance of proteins encapsulated in reverse micelles dissolved in alkane solvents of modest viscosity such as pentane can be directly reproduced in very low viscosity liquefied ethane (Peterson et al., 2005a) and thereby take advantage of the reverse micelle approach as originally envisaged, that is, improve the relaxation properties of the protein such that the full power of solution NMR can be applied. The initial results presented here also provide the foundation for investigation of larger protein complexes associated with membrane-anchored protein cores. It should also be noted that the very simple reverse micelle surfactant system used here has quite different steric and electrostatic properties from a natural membrane bilayer. It is useful to begin consideration of reverse micelle surfactant systems to mimic the basic properties of membrane bilayers and "membrane rafts" (Pike, 2006).

EXPERIMENTAL PROCEDURES

Encapsulation of Recoverin(+Myr) in Reverse Micelles

Myristoylated recoverin was prepared and purified from unmyristoylated protein by HPLC as previously described (Ames et al., 1994). The purified protein was >95% myristoylated as judged by electrospray mass spectrometry. A 0.5 mM solution of [^{15}N]recoverin(+myr) was buffer exchanged and concentrated using a 10K Amicon ultra-15 cell into 10 mM Tris, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EGTA (pH 8.0). The maximum concentration tolerated was ~2 mM before aggregation of the myristoylated protein was evident. The identification of optimal encapsulation conditions for recoverin(+myr) is summarized in the main text. The reverse micelle slurry of 200 mM CTAB, 8.5% (v/v) hexanol in d_{12}-pentane was prepared in a glass vial. The concentrated protein solution (~68 μl) was injected at a calculated W_o of 25 (H_2O:CTAB molar ratio) with a Pipetman into the reverse micelle slurry and vortexed. With the addition of the aqueous protein solution, the reverse micelle assembles and the slurry clears in a matter of a few minutes with only gentle agitation. The solution was transferred to a Wilmad screw cap NMR tube for data acquisition. The reverse micelle solution was 180 μM recoverin(+myr), 200 mM CTAB, 8.5% (v/v) hexanol in d_{12}-pentane. Encapsulated

recoverin(+myr) was titrated with calcium by direct addition of aliquots of 100 mM $CaCl_2$ in water. The sample was briefly vortexed to facilitate mixing.

Encapsulation of the HIV-1 MA(+Myr)

^{15}N-labeled HIV-1 MA(+myr) protein was prepared and purified from unmyristoylated protein as described previously (Massiah et al., 1994; Tang et al., 2004). The purity of the myristoylated protein was confirmed by electrospray mass spectrometry. The ^{15}N-labeled HIV-1 MA(+myr) was buffer exchanged and concentrated using a 15K Amicon ultra-4 cell into 1/10 the NMR buffer concentration: 2 mM sodium phosphate, 4 mM NaCl (pH 6.5). The maximum concentration tolerated was ~0.4 mM before aggregation of the myristoylated protein was evident. A protein aliquot of ~400 μl was then lyophilized. The lyophilized aliquot was then rehydrated in 1/10 the volume, ~40 μl, resulting in a 20 mM phosphate, 40 mM NaCl buffered protein solution. The reverse micelle slurry of 200 mM CTAB, 10% (v/v) hexanol in d_{12}-pentane was prepared in a glass vial. A sufficient volume of the concentrated protein solution (~40 μl) to give a calculated water loading (W_o) of 15 (H_2O:CTAB molar ratio) was injected into the reverse micelle slurry and vortexed. The initially cloudy solution cleared in a few minutes. The reverse micelle solution in d_{12}-pentane was 470 μM HIV-1 MA(+myr), 200 mM CTAB, 10% (v/v) hexanol and was transferred to a Wilmad screw cap tube for NMR data acquisition.

Titration of Encapsulated HIV-1 MA(+Myr) with PI(4,5)P_2 18:0/20:4 Lipid

The titration with PI(4,5)P_2 is somewhat complicated by the lack of solubility of this lipid in either water or alkane solvent. The PI(4,5)P_2 (Avanti Polar Lipids) was first dissolved in $CHCl_3$:MeOH:H_2O 20:9:1 (v/v/v) at a concentration of 600 μM. Aliquots were then dried onto the surface of a glass vial with a stream of argon gas to form a thin film of the lipid. Solutions of encapsulated MA(+myr) in CTAB/hexanol reverse micelles were vortexed in the vial containing the thin film and the lipid was solubilized into the reverse micelle assembly. The titration proceeded in increments of 0.5:1.0 to a final molar ratio of 5.5:1.0. Raising the concentration of lipid above ~1 mM in the 200 mM CTAB solution causes degradation of the reverse micelle solution resulting in extensive precipitation.

NMR Spectroscopy

^{15}N HSQC spectra were collected on Bruker Avance III NMR spectrometers operating at 750 and 600 MHz (^{1}H). Three-dimensional ^{15}N NOESY HSQC spectra were collected on the [^{15}N]recoverin(+myr) and ^{15}N-labeled HIV-1 MA(+myr) reverse micelle samples using an NOE mixing time of 110 ms. The d_{12}-pentane was used to lock the spectrometer. Both spectrometers were equipped with a cryogenically cooled probe that provided excellent signal to noise on the 70–180 μM encapsulated protein solutions owing to their optimal low conductivity (Flynn et al., 2000). FELIX was used to process the data and SPARKY (Goddard and Kneller, 2004) was used for data analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.str.2009.11.010.

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